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ABSTRACT

Objective: Non-obese diabetic (NOD) mice exhibit autoimmune diabetes and Sjögren's-like syndrome. We tested if a therapy that reverses end-stage diabetes in the NOD mouse would affect their Sjögren's-like syndrome.

Methods: NOD mice have a proteasome defect. Improperly selected naïve T cells escape but can be killed by re-introducing MHC class I-self peptides on matched normal splenocytes. The proteasome defect also impairs NFkB, a transcription factor in pathogenic memory T cells, increasing their susceptibility to TNF induced apoptosis stimulated via complete Freund's adjuvant (CFA). We studied the impact of this two-limb therapy (injections of matched normal splenocytes and CFA) on the autoimmune salivary gland disease of the NOD mice.

Results: All NOD mice receiving the above therapy had a complete recovery of salivary flow and were protected from diabetes. Restoration of salivary flow could be a result of a combination of rescue and regeneration of the gland, as confirmed with immunohistochemistry. All untreated NOD mice showed a continuous decline in salivary flow, followed by hyperglycemia and death.

Conclusion: This study establishes that a brief intervention into NOD mice with Sjögren's-like syndrome can reverse salivary gland dysfunction.

Key words: Sjögren's syndrome, salivary gland regeneration, MHC class I, proteasome, Y chromosome lineage tracking.

Sjögren's syndrome is an autoimmune disease that affects between 1-4 million Americans. These patients suffer from the inability to produce saliva, which can profoundly affect their quality of life, interfering with eating, speaking, and sleeping. Treatments to restore saliva production would greatly increase the quality of life for these patients. Possible therapeutic approaches can be tested in the NOD mice which presents a Sjögren's-like syndrome [1]. NOD mice display infiltrates of lymphocytes and a gradual loss of salivary function. The reduced saliva output mimics in part the condition seen in patients with Sjögren's syndrome.

Faustman and coworkers reported that an intervention with complete Freund's adjuvant (CFA) combined with matched MHC class I and self-peptide bearing splenocytes can permanently reverse established end-stage type 1 diabetes in the NOD mouse [2,3]. This two-limb therapy worked by selective apoptosis of two subpopulations of disease causing T cells [4,5]. One limb of this therapy allowed MHC class I and self-peptide complex on matched normal cells (splenocytes) to re-select naïve pathogenic T cells. The second limb induced endogenous TNF-α to kill disease causing activated T cells [5]. Once autoimmunity was removed, the return of normoglycemia in the previously hyperglycemic animals was driven by the regeneration of pancreatic beta cells.

T cell selection occurs by both negative and positive selection through antigen presentation, and in the periphery with the additional aid of cytokines. Antigen presentation through MHC class I and self-peptides allows efficient negative selection (in the bone marrow and thymus) of auto-reactive CD8 T cells in their naïve state. Efficient self-peptide display within the MHC class I groove is driven by the preparation of peptides in the cytoplasm by the LMP2 expressing proteasome. The LMP2 protein is an obligatory subunit for the preparation of peptides for T cell self-tolerance and is encoded in the highest risk region of the genome associated with autoimmunity, i.e. the MHC class II region. After T cells reach the periphery and the T cell receptor is triggered, the LMP2 protein plays an additional role in T cell selection. In highly activated T cells, the proteasome activates NFκB, a transcription factor with multiple roles including apoptosis resistance. NFκB activation protects activated T cells from death after exposures to cytokines such as TNF [5].

NOD mice have a defect in the production of the LMP2 protein and therefore have auto-reactive T cells in the periphery that are both in the naïve and activated state[6,7]. The ability to remove established autoimmunity in this NOD model is by the re-establishment of targeted apoptosis of disease causing T cells. Auto-reactive naïve T cells can be selectively induced to die, even in advanced autoimmune disease, by the reintroduction of the missing MHC class I and self-peptide complexes. Auto-reactive activated T cells can be induced to die by the stimulation of NFκB with low dose TNF.

These fundamental defects in self-tolerance could have a central role in Sjögren's syndrome in humans. Antigen processing genes have previously been implicated in Sjögren's syndrome as well as an observed altered expression of peptide filled MHC class I structures [8,9]. Krause and coworkers[10] reported that Sjögren's syndrome patients have interrupted MHC class I presentation and that their immune cells similarly have LMP2 deficiencies. Therefore humans with Sjögren's syndrome and the NOD mice could have a similar genetic problem resulting in improper T cell selection.

The objective of this study is to assess if a two-limb therapy, which reversed end-stage diabetes in NOD mice by selective killing of pathogenic naïve and activated T cells, could halt or reverse xerostomia (dry mouth) in established Sjögren's-like syndrome (based on the commonality of similar genetic defects in both the mouse and human).

METHODS

Animals. Female NOD (Taconic Farms NY), female C57BL/6, and male CByF1B6F1/J mice (Jackson Laboratory ME) were used as previously described [2]. Complete Freund's adjuvant (Difco MI) was injected into each hind footpad simultaneously with the first splenocyte injection (see supplementary materials and http://www.sciencemag.org/cgi/data/302/5648/1223/DC1/1). Salivary flow rates (SFR), focus score, and blood glucose were measured as described before [11].

Histological Examination. Fluorescence in situ hybridization (FISH) combined with immunohistochemical staining (IHC) was performed as outlined previously [2,12,13]. The primary antibodies used were directed against salivary and pancreatic cell markers and co-localized with a repeat sequence on the mouse Y chromosome [14](supplementary materials).

Statistical analysis. The percentage of normoglycemia was indicated by the Kaplan-Meier method. SFR and focus scores were analyzed with the Mann-Whitney U test.

RESULTS

Salivary flow rates (SFR) of thirteen female NOD mice were monitored. Mice with advanced Sjögren's-like syndrome (>50% SFR loss) were randomized into two groups: untreated (n=5) versus treated NOD (n=8). All NOD mice were normoglycemic at the start of therapy, at 14-week-old. Treated NOD received one injection of CFA for TNF induction and male donor live MHC class I and peptide matched splenocytes bi-weekly for 40 days [2]. The use of male splenocytes allows for post-transplantation tracking of these Y-chromosome cells (using FISH) in female NOD salivary and pancreas tissue. As a control group C57BL/6 mice were similarly studied (n=14).

Female NOD mice exhibit a decrease in SFR at 12-14 weeks of age. SFR were compared between untreated NOD, treated NOD, and age-matched C57BL/6 mice at 10-week (prior to Sjögren's-like syndrome), 14-week (prior to CFA/splenocytes treatment), 21-week (treatment completed), and 35-week of age (sacrifice). All eight treated NOD mice exhibited a decrease in SFR during the 40 days of active therapy (Fig.1A). During the next 120 days, however, there was a gradual restoration of SFR. By 160 days, SFR of treated NOD mice was comparable to that of age-matched C57BL/6 mice ($p=0.6434$). Treated NOD mice were also protected from diabetes ($p=0.0002$; Fig.1B). SFR of untreated NOD mice continued to deteriorate over time, and all of them died of severe hyperglycemia within 140 days from the start of therapy. SFR directly reflects function of the glands and its decrease is the major clinical finding in patients with Sjögren's syndrome.

To investigate the mechanism of the return of SFR in treated NOD mice, we examined the salivary tissues histologically for chimerism and inflammatory signs. Restoration of SFR could be a result of rescue of a damaged gland or a combination of rescue and regeneration. Regeneration could derive from endogenous salivary cells (after being liberated from T cell suppression) and/or from donor splenic precursor cells. We

combined FISH with IHC in the same section to allow for the simultaneous detection of both the Y chromosome and specific markers for salivary or pancreatic cells. A small number of Y-positive salivary epithelial cells (Fig.2) and a few pancreatic beta cells were detected in the treated NOD (data not shown), but not in untreated NOD or C57BL/6 mice. We observed the reappearance of pancreatic islets free of invasive lymphoid infiltrates in treated NOD mice (data not shown). However, in salivary glands, the lymphocytic infiltrates (focus score) did not differ significantly between splenocyte-treated and untreated animals ($p=0.23$).

DISCUSSION

We used 14-week-old normoglycemic NOD mice with established Sjögren's-like syndrome. The successful treatment criteria were: 1) preventing progression to diabetes, 2) islet regeneration or rescue, 3) restoration of SFR, and 4) salivary tissue regeneration. Our results showed 100% protection from progression to diabetes when this therapy was administered in young (14-week-old) NOD mice. This percentage is higher than the 85% success rate reported by the Faustman's group [2.] for end-stage diabetic NOD at 22- to 40-week-old, suggesting the importance of early intervention. Lineage tracking methods [15] confirmed that although both islet rescue from the recipient and islet regeneration from donor injected splenocytes occurred; in young animals the islet rescue (or host-derived regeneration) dominated. We demonstrate that salivary gland function, as measured by SFR, was completely restored. A small number of donor splenocytes colonized the salivary gland and differentiated into salivary epithelial cells.

Unlike the pancreas, the salivary glands of treated mice did not show a change in focus score (lymphoid infiltrates within the glands). In the pancreas of these mice, the"benign" circumferential insulitis increased and this patterning is known in the NOD model to never progress to disease. In the pancreas, the aggressive (active) invasive insulitis never appeared after treatment. Unfortunately in the salivary gland, this patterning of salivary gland infiltrates does not distinguish between "benign" and active disease. This explanation may also be valid in human Sjögren's Syndrome where SFR did not correlate with the focus score of minor salivary gland biopsies[16.].

This study demonstrates that a two-limb intervention can stably reverse two forms of established autoimmune disease i.e. diabetes and xerostomia in Sjögren's-like syndrome. With the recent finding that humans with Sjögren's syndrome have identical proteasome defects in their LMP2 subunit as the NOD mice[10.], Sjögren's syndrome patients might indeed benefit from this therapy.

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There are no competing interests from all authors.

FIGURE LEGENDS

Fig. 1. Effects of treatment on the restoration of salivary flow rates (SFR) and maintenance of normoglycemia in autoimmune prone NOD mice. (A) Stimulated salivary flow rate (+/- SEM) of treated NOD (open circles), untreated NOD (crosshatch) and control C57BL/6 mice (solid squares) during the 160 day time course. The bar indicates the 40-day treatment period. (B) Kaplan-Meier plot for normoglycemia for treated NOD (open circle) compared to untreated NOD (cross hatch).

Fig. 2. Double immunostaining of a section of a salivary gland of a successfully treated female NOD mouse (A) is shown in three planes restored from a Z-stack of sections taken at 0.5 µm intervals. The Y chromosome signal is green and cytokeratin13 (a marker of salivary epithelial cells) is red. Nuclei are stained in blue with DAPI. We observed Y-chromosome positive nuclei in cytokeratin-positive cells of several treated female NOD mice. The Y and Z dimensions are shown on the two sides of the X plane image and demonstrate the presence of the Y chromosome (green) in the same plane with the cytokeratin13 (red) and the nucleus (DAPI-blue). FISH analysis of salivary tissue sections from untreated female (B) and male (C) mice are used here as controls for the Y chromosomal probe.

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ONLINE SUPPLEMENTARY MATERIALS

Mice and spleen cells

Female NOD mice (Taconic Farms, Germantown, NY), female aged-matched C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), and male CByF1B6F1/J (CByB6F1) mice (Jackson Laboratory) were maintained under pathogen-free conditions.

Matched normal splenocytes of male CByB6F1 mice ($9x10^6$ cells) were harvested and injected into female NOD recipients through the tail vein twice a week for 40 days, as previously described [2]. Complete Freund's adjuvant (CFA, Difco, Detroit, MI) was freshly mixed with an equal volume of physiological saline and then injected (50 µl) into each hind footpad simultaneously with the first splenocyte injection.

Monitoring disease progression

Secretory function of the salivary glands (salivary flow rate). Measurement of salivary flow rates were obtained by inducing mild anesthesia to NOD and C57BL/6 mice with 1 µl/kg body weight of a 60 mg/ml ketamine (Phoenix Scientific, St. Joseph, MO, USA) and 8 mg/ml xylazine (Phoenix Scientific) solution given intramuscularly [11]. Whole saliva was collected after stimulation of secretion using 0.5 mg pilocarpine/kg body weight administered subcutaneously. Saliva was obtained from the oral cavity by micropipette, placed into pre-weighed 0.5-ml microcentrifuge tubes. Saliva was collected for a 20-minute period and its volume determined gravimetrically.

Diabetes. Body weights and blood glucose concentrations from NOD mice were monitored three times a week (Glucometer Elite instrument, Bayer, Mishawaka, IN, USA). NOD females were diagnosed with diabetes after observing weight loss accompanied by two consecutive blood glucose concentrations of >400 mg/dl.

Histological Examination

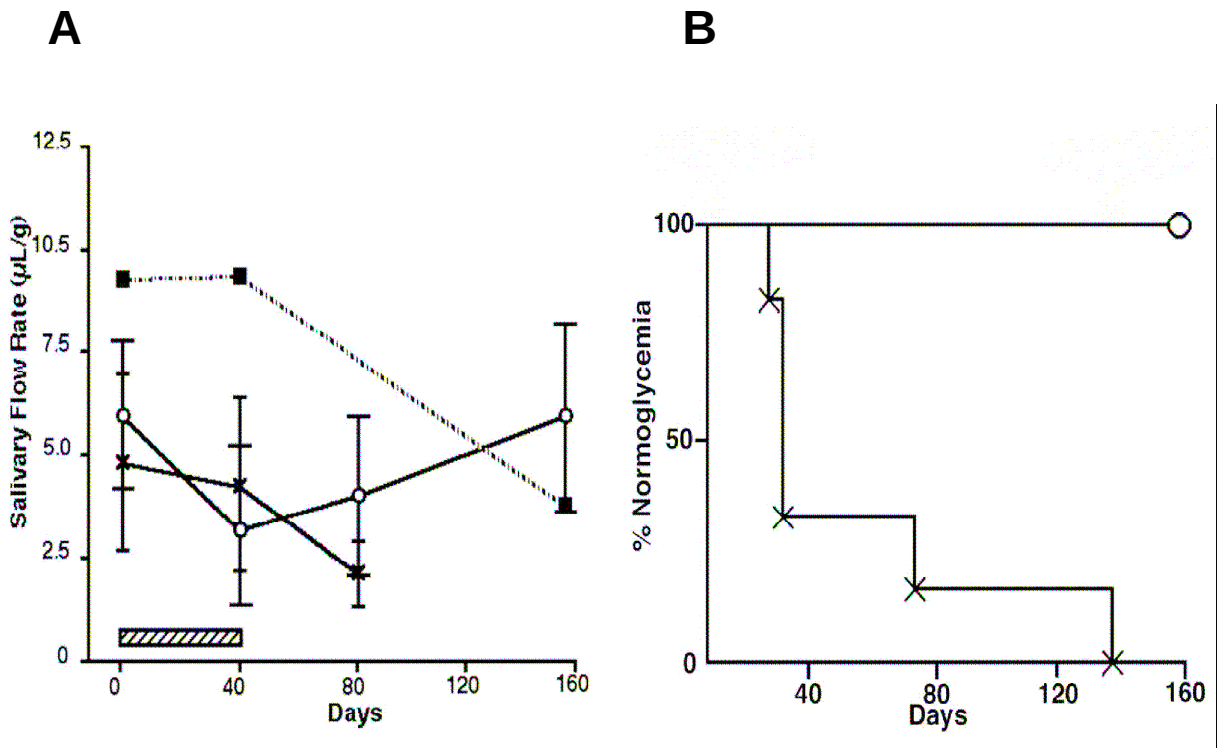
Fluorescence in situ hybridization (FISH) combined with immunohistochemical staining (IHC). This method was performed as outlined previously [2, 12, 13]. In brief, salivary gland and pancreas were collected, immersed in OCT compound, and then frozen at –80°C. Serial frozen sections were cut in a cryostat at 5-µm thickness and fixed in a 4% picric acid-aldehyde fixative for 15 minutes. Immunostaining was performed by primary antibodies that were detected by the Sternberger peroxidase antiperoxidase (PAP) method followed by a FITC-tyramide signal amplification (TSA System, Invitrogen,Carlsbad, USA). The primary antibodies used were directed against salivary and pancreatic cell markers. We used an antibody to cytokeratin 13 (BioGenex, San Ramon, USA) to detect salivary epithelial cells and to insulin (Bachem Bioscience, King of Prussia, USA, cat# T-5014.0400) to detect pancreatic beta cells. After immunostaining was developed using tyramide conjugated to AlexaFluor 594, FISH was performed on the same tissue section to detect the Y chromosome. A digoxigenin-labeled riboprobe was generated from a template that recognizes a repeat sequence (pY353B) on the mouse Y chromosome [14]. The riboprobe was then detected using an antibody to digoxigenin conjugated to peroxidase (Roche, Indianapolis, USA). This peroxidase was visualized by tyramide signal amplification with the FITC fluorochrome-tyramide reagent (Invitrogen). All sections were stained with DAPI (Sigma) and mounted with 80% glycerol/ 20% Tris. Finally the slides were visualized using a Leica DMX6000 inverted fluorescent microscope equipped with a motorized stage, and images were captured using a Hamamatsu camera. For confocal imaging, Z stacks were taken at an interval of 0.5 µm

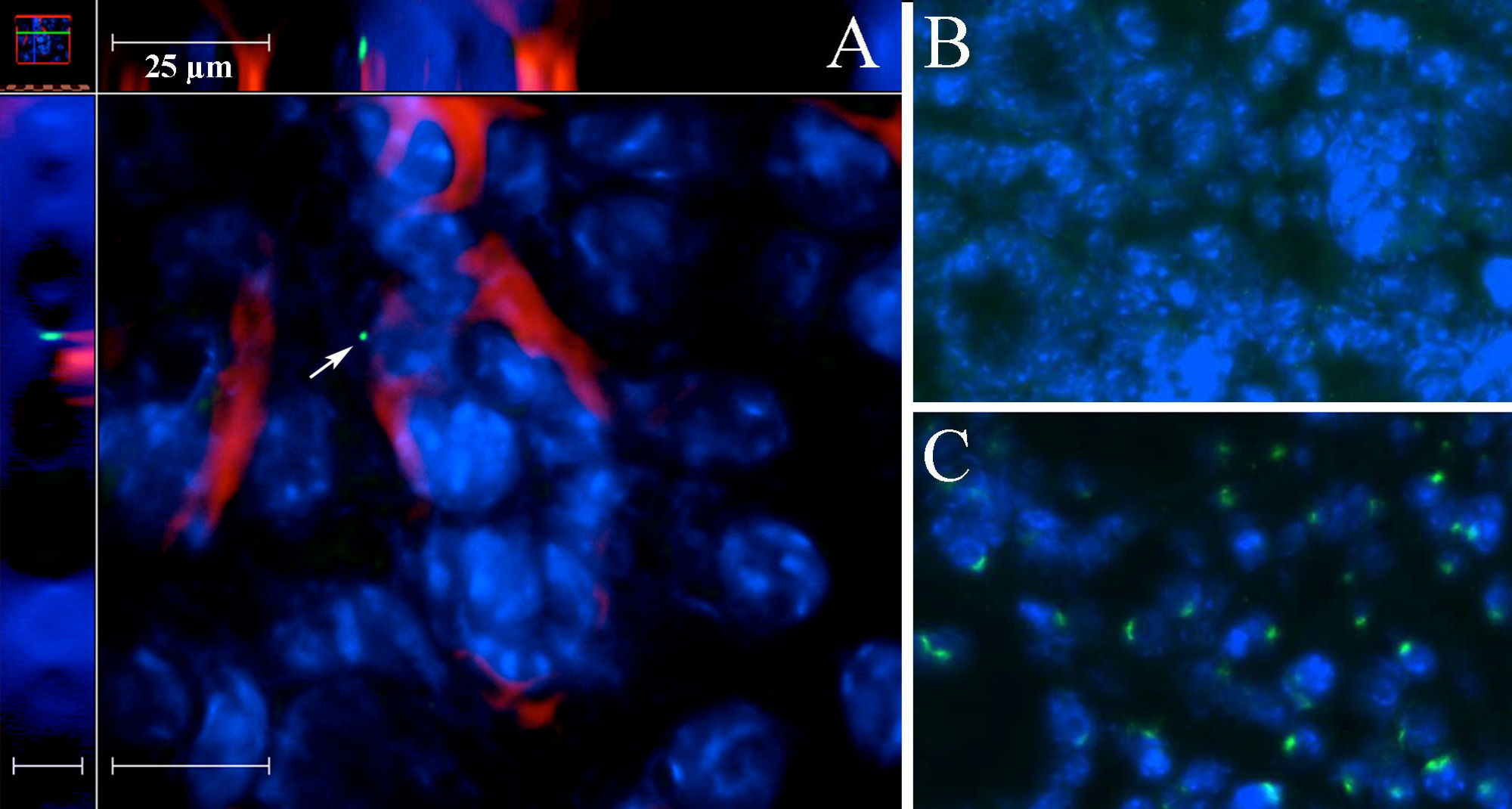
and iterative restoration was performed at 90% confidence and 25 iterations using Volocity software.

Focus score. NOD mice were sacrificed, and their submandibular glands immediately removed. One gland of each mice was fixed in 10% formalin and embedded in paraffin. Sections were cut at 5 μm thickness and subsequently stained with hematoxylin and eosin. A focus is defined as an aggregate of 50 or more lymphocytes and histiocytes per 4 mm sq. of histologic section. This method is an accepted way to determine the severity of the sialadenitis (the inflammatory cell infiltrate that is found in the salivary glands).

SUPPLEMENTARY FIGURE

Fig S1. Salivary glands of successfully treated female NOD mice (A- D) are shown. The Y chromosome signal is green and cytokeratin13 (a marker of salivary epithelial cells) is red. Nuclei are stained in blue with DAPI. We observed Y-chromosome positive nuclei in cytokeratin-positive cells of treated female NOD mice. The Y and Z dimensions are shown on the two sides of the X plane image and demonstrate the presence of the Y chromosome (green) in the same plane with the cytokeratin13 (red) and the nucleus (DAPI-blue). These four images (A, B, C, D) were taken from two different treated NOD mice. FISH analysis of salivary tissue sections from control female (E) and male (F) mice are used here as controls for the Y chromosomal probe.

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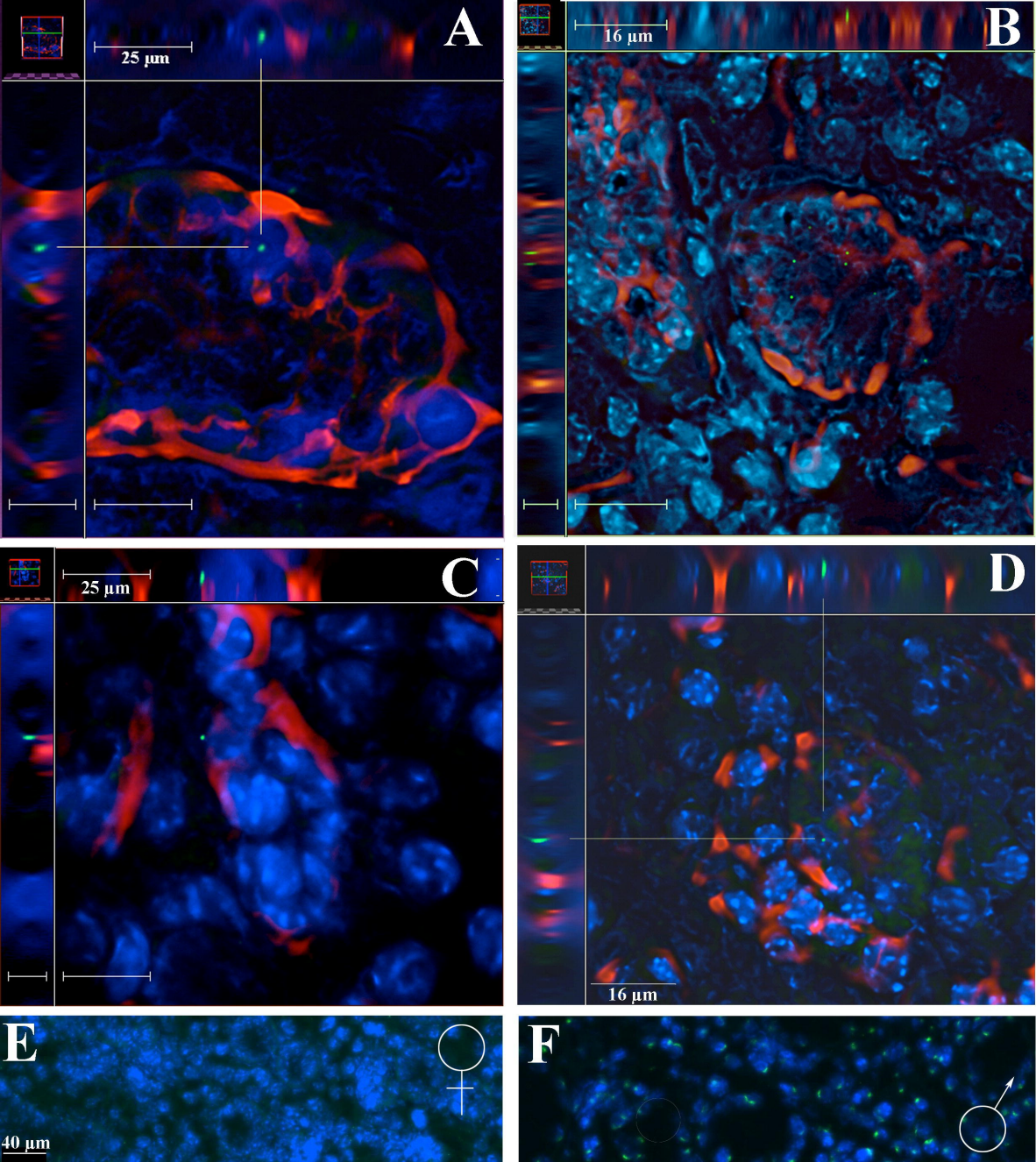
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